



Synthesis of a conductive network of crosslinked carbon nanotube/hemoglobin on a thiol-modified Au Surface and its application to biosensing



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ABSTRACT

The synthesis of a network of crosslinked carbon nanotube/hemoglobin (CNT/Hb) on a thiol-modified Au surface and its use as sensor for H₂O₂ is reported. The constructed CNT/Hb 3-D network exhibits high conductivity by expediting the electrical transfer from Hb to the Au electrode and has a high electrocatalytic property for sensing hydrogen peroxide (H₂O₂). CNTs were first oxidized by treatment with concentrated nitric acid for 10 h and functionalized by reaction with the thiol group of 4-aminothiophenol and Hb was also reacted with 4-aminothiophenol. These modified CNTs and modified Hb were immobilized on a 4-aminothiophenol monolayer-modified Au electrode by co-electropolymerization by repetitive cyclic voltammetry scans ranging between -0.1 and $+1.1$ V (versus Ag/AgCl). Cyclic voltammogram (CV) and amperometry were employed to study electrochemical properties of the modified electrodes. Direct electrical communication between the redox center of Hb and an Au electrode was established through 3-D network of crosslinked CNT/Hb. The Hb present in the 3-D CNT/Hb network exhibited a pair of quasi-reversible redox peaks with a midpoint potential of -0.225 V and -0.075 , cathodic and anodic respectively. The electron transfer rate constant, k_s and electron transfer co-efficient α were found to be 0.51 s⁻¹ and 0.58 , respectively. The modified electrode was used as a biosensor and exhibited a high sensitivity, long linear range and lower detection limit to H₂O₂, under optimal conditions. The apparent Michaelis–Menten constant (K_m) and Hb adsorption in the CNT/Hb network with average surface coverage of were found to be 0.19 mM and 4.8×10^{-10} mol cm⁻², respectively. This system should be very useful for other sensing applications.

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1. Introduction

A fundamental requirement of any biosensor/bioelectronics system is to establish electronic coupling and good communication between the biomolecules (e.g., enzyme, protein) and the electronic supports (electrode). The sensing principle of such a bioelectronics device is based on the ability of the enzyme to catalyze a selective transformation of a specific substrate. The information then has to be conveyed to the read-out component of the system as an electrical signal. Electrical contact between biomolecules (e.g., redox enzyme) is the challenging task since the redox centers of the enzyme are located deep inside the insulated shells. The lack of good electrical communication between the biomaterial components and the electronic elements has been a major obstacle in a number of recent studies (Wang, 2008; Willner and Katz, 2005; Liu et al., 2007). Therefore, the main issue in bioelectronics is to ensure the electrical coupling of

biomolecules with electrodes to facilitate direct electron exchange and that is the challenge addressed in the present work.

Direct electrical wiring between electron-relay modified enzymes and metal electrodes has opened a new route to biosensor and bioelectronics devices and to the direct electrochemical synthesis of biochemicals is the fundamental interest of many researchers (Willner et al., 1997). Since redox enzymes do not exchange electrons with simple metal electrodes in homogenous solution, a special fabrication is required to flow current through metal electrodes. Several techniques have been proposed to establish the electrical communication between the redox center of an enzyme and conventional metal electrodes (Raitman et al., 2002; Wang et al., 2006; Yan et al., 2008). In order to fabricate highly sensitive biosensor devices, the generation of high turnover rates between the redox enzyme and electrode surface is very important. Unfavorable orientation or direct adsorption of biomolecules onto a metal electrode surface may dramatically lower their catalytic activity, limiting the charge transport across matrices (Zhang and Li, 2004). In this context, controlled orientation of redox enzyme on an electrode surfaces and their effective electrical wiring have been demonstrated by the application of the reconstitution technique

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(Holland et al., 2011; Zayats et al., 2008; Willner et al., 2009; Fruk et al., 2009). Based on the reconstitution method, the electrical wiring of redox enzyme was accomplished with relay cofactor, metal nanotube/cofactor or nanoparticle/cofactor monolayer-functionalized electrode (Willner and Willner, 2001; Katz et al., 1997; Xiao et al., 2003; Patolsky et al., 2004; Holland et al., 2011). In spite of achieving high electron-transfer turnover rates in those systems, the forming of monolayer of enzyme on the surface of electrodes is a major disadvantage to the method, as a result of the low content of enzyme associated with the surface. All of these results come from the weak contact of the biomolecules to the electrode which causes the low electrical communication for biosensors.

One attractive solution to overcome all of those drawbacks is the use of nanomaterials to give 3D structure to biomolecules for electronic coupling and communication between the biomolecules and the electrode (Yehezkeili et al., 2009). For example, Willner et al. have demonstrated an excellent method to generate three-dimensional electrically wired Glucose Oxidase (GOD) electrodes that showed high electron-transfer turnover rates (Baravik et al., 2009). In this system, thioaniline-modified GOD in the presence of thioaniline-functionalized carbon nanotube (CNT) has created a three-dimensional bis-aniline-crosslinked CNT/GOD network. The resultant bis-aniline crosslinked network was redox active and able to mediate the electron from redox site of GOD to the base electrode. However, in spite of the successful demonstration of GOD wiring with electrode, there is no report on effective creation of a Hemoglobin (Hb)/CNT 3-D network. Nanoscale surfaces such as nanotubes or nanoparticles can overcome some limitations because they contain regions of high curvature that energetically limit biomolecules adsorption, and reduced steric hindrance of the substrate to binding site on the enzyme. The unique electrical properties, high chemical stability and high surface-to-volume ratio of carbon nanotubes have been intensively used for studying electron transfer of enzyme/protein for biosensor design and other purposes (Gooding et al., 2003; Yang et al., 2007; Sardesai et al., 2011). The ability of CNT to promote the electron-transfer reactions of enzyme such as Horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) suggests great promise for enzyme-based amperometric biosensors (He and Dai, 2007). Redox enzymes can be immobilized on CNT with enhanced retention of their biocatalytic activity and used different biosensor devices by associating with transistors and electrode (Gooding et al., 2003). CNTs functionalized with redox enzyme associated with electrodes show high surface areas, effective charge transport and good electrical contact and led to use in biosensing and biofuel cell (Yehezkeili et al., 2009; Wang, 2005a,b). Biosensors have major advantages over traditional analytical methods, which has resulted in their more pronounced use in the biomedical and environmental fields (Wang et al., 2005). Significant progress has been made in the development of sensors, particularly in terms of sensitivity, selectivity, stability and simplicity (Dorst, et al., 2010; Kafi et al., 2008, 2010; Ligler, 2009; Kotani et al., 2011).

Herein, we have focused on a construction of three-dimensional (3-D) conductive Hemoglobin network with CNT onto the thiol-modified gold (Au) electrode. In order to obtain the 3-D network, CNTs modified with electropolymerizable aniline and 4-aminothiophenol-modified Hemoglobin were coelectropolymerized on the thiol-modified Au electrode surface. Our results show that the created 3-D CNT/Hb composite effectively builds a good electrical communication between the redox centers of the Hb and an electrode. We have also evaluated the resultant 3-D CNT/Hb network in H_2O_2 biosensing as the sensitive and accurate H_2O_2 detection is of great interest to researchers because of its importance in the pharmaceutical, clinical and industrial settings (Wang and Wang, 2004).

2. Experimental

2.1. Reagents and materials

Hemoglobin was purchased from Sigma, used as received and stored at 4 °C. *N*-(ϵ -Maleimidocaproyloxy) sulfo succinimide ester (sulfo-EMCS) was purchased from PIERCE (Through Thermofisher). Single-walled carbon nanotubes, 4-aminothiophenol, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Aldrich. *N*-hydroxysulfo succinimide sodium salt (NHS) was obtained from Sigma. 30% Hydrogen peroxide was bought from Fluka and the stock solution of H_2O_2 was diluted from this. All experimental solutions were prepared fresh by appropriate dilution of the stock solutions. All other reagents were of analytical grade and used as supplied. The pure water (18 M Ω cm) used to prepare all solutions in this study was purified with the Nanopure[®] water system. All experiments were performed in 0.1 mol l⁻¹ phosphate buffer solutions (PBS) whose pH values were adjusted with K_2HPO_4 and KH_2PO_4 .

2.2. Modification of Hb

Hemoglobin was functionalized based on the method described elsewhere (Baravik et al., 2009). Hb (50 mg) was dissolved in 0.1 M phosphate buffer (3 mL, pH 7.4). The solution was then be treated with *N*-(ϵ -Maleimidocaproyloxy) sulfo succinimide ester (sulfo-EMCS) and stirred for 40 min. Then the solution was mixed with 4-aminothiophenol (thioaniline) and left for 2.5 h. Finally, the solution was eluted through a G-25 column (GE Healthcare) with 0.1 m phosphate buffer (pH 7.4) as the eluent. The resulting purified, functionalized Hb solution was lyophilized to yield a powder that was stored at -20 °C.

2.3. Modification of CNT

Prior to modification, CNTs were sonicated for 10 h in a mixture solution of concentrated sulfuric acid and nitric acids (3:1), resulting the oxidization of CNTs. The oxidized CNTs were washed with water and centrifuged several times to a final pH of 7.0. The CNTs were then reacted with a HEPES buffer, 0.01 M, pH=7.4, containing 2-(4 aminophenyl)-ethylamine and was stirred with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfo succinimide sodium salt (NHS) for 2 h at room temperature. Finally, the modified CNTs were thoroughly rinsed with water and centrifuged, at least four times, using a PBS solution.

2.4. Formation of 3-D CNT/Hb network on 4-aminothiophenol-modified Au

A gold (Au) electrode (2 mm diameter) was used in the formation of 3-D CNT/Hb network for this work. At first, the Au electrode was electrocycled ranging between -0.1 and +1.1 V (versus SCE) in solution containing H_2SO_4 to remove any contaminations and immersed in ethanol before monolayer assembly. The cleaned Au-electrode was modified by immersion in a solution of 50 mM thioaniline in ethanol. After 24 h to ensure a complete coverage, the Au electrode was thoroughly rinsed with ethanol and dried under a stream of nitrogen. This modified Au-electrode was then electropolymerized with modified CNTs and Hb in 0.1 M phosphate buffer (pH 7.4) by using a fixed number of repetitive cyclic voltammetry scans, ranging between -0.1 and +1.1 V (versus SCE), at a scan rate of 100 mV s⁻¹. The resulting modified Au electrode is denoted below as Au/SAM/CNT/Hb. For comparison, another electrode Au/SAM/Hb was also modified.

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